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PRINCIPAL INVESTIGATOR: Arthur M. Mercurio, Ph.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center, Inc.
Boston, Massachusetts 02215

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Arthur M. Mercurio, Ph.D.

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The α6β4 integrin promotes breast carcinoma invasion by its activation of a phosphoinositide 3-OH (PI3 K)signaling pathway (Shaw et al., Cell 91:949-960). We demonstrate here using MDA-MB-435 breast carcinoma cells that α6β4 stimulates chemotactic migration, a key component of invasion, but that it has no influence on haptotaxis. Stimulation of chemotaxis by α6β4 expression was observed in response to either lysophosphatidic acid (LPA) or fibroblast conditioned medium. Moreover, the LPA-dependent formation of lamellae in these cells is dependent upon α6β4 expression. Both lamellae formation and chemotactic migration are inhibited or 'gated' by cAMP and our results reveal that a critical function of α6β4 is to suppress the intracellular cAMP concentration by increasing the activity of a rolipram-sensitive, cAMP-specific phosphodiesterase (PDE). This PDE activity is essential for lamellae formation, chemotactic migration and invasion based on data obtained with PDE inhibitors. Although PI3-K and cAMP-specific PDE activities are both required to promote lamellae formation and chemotactic migration, our data indicate that they are components of distinct signaling pathways. The essence of our findings is that α6β4 stimulates the chemotactic migration of breast carcinoma cells through its ability to influence key signaling events that underlie this critical component of breast carcinoma invasion.

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Seth H. Munn 10/19/98

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5) INTRODUCTION (Note: the work accomplished during Year 2 of this grant focused on Aims 2 and 4 of the original application).

Breast carcinoma invasion is a complex process that involves directed migration and localized proteolysis (23). Although the mechanistic basis of invasion had been elusive, recent advances in molecular cell biology have facilitated a much more rigorous analysis of this important and critical component of cancer progression. In particular, insight into the function and regulation of cell adhesion receptors, as well as proteases, has fueled significant progress in our understanding of the invasive process. Studies aimed at defining specific signal transduction pathways that determine the behavior of invasive carcinoma cells are also contributing to an uncovering of the molecular basis of invasion.

Recent work by our group and others has implicated a key role for the α6β4 integrin in carcinoma invasion (3, 10, 31, 34, 39, 46). This integrin, which is a receptor for the laminins, is essential for the organization and maintenance of epithelial structure. In many epithelia, α6β4 mediates the formation of stable adhesive structures termed hemidesmosomes that link the intermediate filament cytoskeleton with the extracellular matrix (2, 12). The importance of this integrin in epithelial structure has been reinforced by the generation of β4-nullizygous mice that exhibit gross alterations in epithelial morphology and anchorage to the basement membrane (9, 45). In contrast to its function in normal epithelia, α6β4 can stimulate carcinoma migration and invasion through its ability to interact with the actin cytoskeleton and mediate the formation and stabilization of lamellae (31). This dynamic function of α6β4 in enhancing the migration of invasive carcinoma cells is quite distinct from its role in maintaining stable adhesive contacts in normal epithelia by associating with intermediate filaments. In fact, we have established that the ability of α6β4 to stimulate carcinoma migration and invasion depends upon its preferential activation of a PI3-K/Rac signaling pathway that is necessary for invasion (39). In essence, our studies have defined an integrin-mediated mechanism of carcinoma invasion that involves the stimulation of carcinoma migration by the dynamic association of α6β4 with F-actin and the activation of a specific signaling pathway by this integrin.

Although we have established the involvement of α6β4 in the migration of invasive breast carcinoma cells, the nature of this migration has not been well-defined. Moreover, signaling pathways distinct from PI3-K/Rac that are also regulated by α6β4 are likely to contribute to carcinoma migration. For these reasons, we sought to examine the migration mediated by α6β4 in more detail and to identify other signaling pathways regulated by this integrin that contribute to migration. The results obtained indicate that α6β4 stimulates the chemotactic migration of invasive carcinoma cells but that it has no influence on their haptotactic migration. Importantly, we demonstrate that the ability of α6β4 to suppress the intracellular cAMP concentration ($[cAMP]_i$) by activating a cAMP-specific phosphodiesterase is essential for its enhancement of lamellae formation and chemotactic migration. Although PI3-K and cAMP-specific phosphodiesterase activities are required for lamellae formation and chemotactic migration, we conclude that they are components of distinct signaling pathways.

6) BODY

Expression of the α6β4 integrin in MDA-MB-435 cells enhances their chemotactic migration: The possibility that expression of the α6β4 integrin influenced the rate of either haptotactic or chemotactic migration was assessed. For this purpose, stable transfectants of MDA-MB-435 cells were used that expressed either the α6β4 integrin (MDA/β4) or a deletion mutant of α6β4 (MDA/β4-ΔCYT) that retains only four amino acids of the β4 cytoplasmic domain, immediately proximal to the transmembrane domain (39). As shown in Figure 1A, subclones of the MDA/β4 transfectants (5B3, 3A7) exhibited a rate of haptotactic migration toward laminin-1 that was slightly lower than the rate observed for subclones of the mock transfectants (6D7, 6D2). In marked contrast, expression of α6β4 induced a substantial increase in the rate of chemotaxis of these cells towards conditioned medium from NIH-3T3 cells (Figure 1B). The rate of chemotaxis of the MDA/β4 transfectants (5B3, 3A7) was 15-20 fold greater than that of the mock transfectants

(6D7, 6D2) over a 4 hr. time period. These data indicate that expression of α6β4 potentiates chemotactic migration of MDA-MB-435 cells without substantially altering their rate of haptotaxis.

To identify specific factors that could cooperate with α6β4 to promote chemotaxis of MDA-MB-435 cells, we tested several growth factors known to have chemotactic potential including epidermal growth factor, basic fibroblast growth factor, hepatocyte growth factor/scatter factor, insulin-like growth factor type I, transforming growth factor α and β, platelet-derived growth factor (AA and BB), somatostatin, thrombin and lysophosphatidic acid (LPA). Of these factors, only LPA was able to mimic the chemotactic effects of NIH-3T3 cell conditioned medium on the MDA-MB-435 transfectants (Figure 1C and data not shown). LPA stimulated the chemotaxis of MDA-MB-435 cells in a dose dependent manner with maximal stimulation observed at 100 nM. Of note, LPA-stimulation of chemotaxis was 5 to 7 fold greater in the MDA/β4 transfectants than in the mock transfectants. Subclones of the MDA/β4 -ΔCYT transfectants (Δ3C12, Δ1E10) exhibited a rate of chemotaxis that was similar to the mock transfectants (Figure 1D) indicating that the β4 cytoplasmic domain is critical for mediating the increased chemotaxis seen in the MDA/β4 transfectants.

The increased chemotaxis observed for the MDA/β4 transfectants in response to LPA was evident on both collagen type I (Figure 1 C, D) and laminin-1 (data not shown), indicating that α6β4-enhanced migration is independent of the matrix protein used for traction. This possibility was examined further by pre-incubating the MDA/β4 transfectants with function-blocking mAbs before their use in the chemotaxis assays. As shown in Figure 2A, inhibition of α6 integrin function with the mAb 2B7 did not block the chemotaxis of the MDA/β4 transfectants on collagen I towards LPA. However, this mAb inhibited the haptotaxis of MDA-MB-435 cells toward a laminin-1 gradient (Figure 2B), a process that is dependent on the α6 integrin (37). Chemotaxis toward LPA was inhibited completely, however, by pre-incubating the cells with the β1 integrin-specific mAb 13 (Figure 2A). Collectively, these data indicate that the stimulation of chemotaxis by expression of α6β4 can be independent of the adhesive functions of α6β4, and that the adhesive interactions required for α6β4-enhanced chemotaxis on collagen are mediated through β1 integrins.

Expression of the α6β4 integrin is required for the formation of lamellae in response to LPA: Chemotactic migration frequently involves the formation of broad sheets of polymerized actin at the leading edge of the cell termed lamellae (26). To determine if expression of the α6β4 integrin influenced the formation of such motile structures, we analyzed the morphology of the MDA-MB-435 transfectants plated on collagen I (Figure 3). Prominent lamellae were not evident in the mock transfectants and treatment with 100 nM LPA did not stimulate a significant increase in lamellar area (Figure 3C, D). The MDA/β4 transfectants exhibited a similar morphology to that of the mock transfectants when plated on collagen (compare Figure 3A to 3C) or laminin-1 (data not shown). Within minutes after LPA treatment, however, the MDA/β4 transfectants formed large, ruffling lamellae (Figure 3C). Quantification of these cells by digital image analysis indicated that LPA stimulated a dramatic increase in the lamellar area of the two subclones of the MDA/β4 transfectants (Figure 3D). In contrast, no increase in the lamellar area of the mock transfectants in response to LPA was detected by this analysis (Figure 3D).

Pharmacological evidence for the involvement of cAMP in chemotaxis: LPA is a bioactive phospholipid that can mediate its effects on cells through a receptor linked to heterotrimeric G-proteins, including inhibitory-type G (Gi) proteins (28). To assess the possible involvement of a Gi protein in α6β4-enhanced chemotaxis, we used pertussis toxin, which inactivates heterotrimeric Gi-proteins by ADP ribosylation (30). The LPA-stimulated chemotaxis of both the MDA/β4 and mock transfectants was inhibited by pertussis toxin with maximal inhibition observed at 100ng/ml (data not shown). These data suggested that the α6β4 integrin enhances chemotaxis that is mediated through pertussis toxin-sensitive, Gi-linked receptors. Gi proteins are known to inhibit certain classes of adenyl cyclases and thus limit cAMP production (44). For this reason, we analyzed the impact of stimulating cAMP production on chemotaxis

using forskolin. Although forskolin inhibited LPA-stimulated chemotaxis, the MDA/β4 and mock transfectants differed significantly in their response to this activator of adenyl cyclases. LPA-stimulated chemotaxis of the mock transfectants was inhibited to basal levels by 50 μM forskolin (Figure 4A). At this concentration of forskolin, the inhibition of chemotaxis of the MDA/β4 transfectants was only 50% and higher concentrations of forskolin (100 μM) did not abrogate chemotaxis of these cells (Figure 4A). Interestingly, treatment of the MDA/β4 or mock transfectants with forskolin did not inhibit haptotactic migration on laminin (Figure 4B). These data indicate that a cAMP-sensitive pathway plays a key role in LPA-stimulated chemotaxis of MDA-MB-435 cells and they suggest that the α6β4 integrin may regulate this pathway.

Expression of the α6β4 integrin in MDA-MB-435 cells influences cAMP metabolism: To determine if α6β4 expression influences the intracellular cAMP concentration ($[cAMP]_i$), the $[cAMP]_i$ was determined in extracts obtained from subconfluent cultures of MDA/mock, MDA/β4 and MDA/β4-ΔCYT transfectants using a cAMP enzyme-linked immunoabsorption assay. As shown in Figure 5A, the MDA/β4 transfectants had a 30% lower $[cAMP]_i$ (2.7 pmol cAMP per 10^6 cells) than either the mock (3.7 pmol cAMP per 10^6 cells) or α6β4-ΔCYT transfectants (3.8 pmol cAMP per 10^6 cells). This difference was statistically significant ($p<0.001$). Of note, neither clustering of α6β4 using the 2B7 mAb and an appropriate secondary Ab nor LPA treatment reduced cAMP levels further (data not shown).

The observation that the MDA/β4 transfectants were more resistant to forskolin inhibition of chemotaxis than the mock transfectants (Figure 4) suggested that these two populations of cells differ in their ability to metabolize the cAMP generated in response to forskolin stimulation. This possibility was examined by determining the $[cAMP]_i$ in forskolin-treated cells. As shown in Figure 5B, the MDA/β4 transfectants exhibited a 30% lower $[cAMP]_i$ than the mock transfectants when plated on collagen I. With forskolin stimulation, a 2.5-fold greater accumulation of cAMP was observed in the mock transfectants (6.6 pmol/10^6 cells) compared to the MDA/β4 transfectants (2.6 pmol/10^6 cells). When the forskolin-treated cells were also treated with the phosphodiesterase (PDE) inhibitor, IBMX, to prevent breakdown of cAMP, the MDA/β4 transfectants exhibited a $[cAMP]_i$ comparable to the mock transfectants (120±11 vs. 104±18 pmol/10^6 cells, respectively; Figure 5C). Together, these data suggest that expression of α6β4 integrin suppresses the $[cAMP]_i$ by increasing PDE activity.

To establish more directly that expression of the α6β4 integrin can regulate cAMP-dependent PDE activity, the activity of this enzyme was assayed in cell extracts obtained from the MDA/mock and MDA/β4 transfectants. As shown in Figure 6A, the MDA/β4 transfectants exhibited a significantly higher rate of PDE activity than the mock transfected cells. Moreover, the PDE activity of the MDA/β4 transfectants was markedly increased (51% for 5B3 and 45% for 3A7) in response to forskolin stimulation compared to the mock transfectants (29% for 6D7; Figure 6A). The difference in PDE activity between the MDA/β4 and mock transfectants was eliminated by rolipram, a type IV PDE-specific (PDE 4) inhibitor (Figure 6B). These data indicate that a cAMP-dependent PDE 4 activity is influenced by α6β4 expression in MDA-MB-435 cells. Also, this activity is likely responsible for the observed decrease in $[cAMP]_i$ and the resistance to forskolin-mediated inhibition of LPA chemotaxis observed in the MDA/β4 transfectants.

To examine the possibility that the MDA/β4 and mock transfectants differed in their level of PDE expression, we assessed PDE 4 expression in these cells using antibodies specific for the various PDE 4 variants (15). The predominant PDE 4 variant expressed in MDA-MB-435 cells is PDE 4B based on results obtained with antibodies specific for PDE 4A, 4B and 4D (data not shown). Importantly, the expression of PDE 4B did not differ significantly between the MDA/β4 and mock transfectants (Figure 6C). These data indicate that the increased PDE activity observed in the MDA/β4 transfectants is not the result of increased PDE expression.

PDE activity is necessary for chemotaxis, invasion and lamellae formation: The importance of PDE for chemotactic migration was examined by treating the MDA/mock and β4 transfectants with IBMX prior to their use in the chemotaxis assay. As shown in Figure 7A,

IBMX inhibited LPA-stimulated chemotaxis with maximal inhibition observed at 1mM. Similar results were obtained with the cAMP-specific PDE inhibitor, rolipram (data not shown). We also examined the involvement of PDE in carcinoma invasion by treating cells with IBMX prior to their use in a standard Matrigel invasion assay. A substantial inhibition of invasion was observed in the presence of IBMX in comparison to the solvent control (Figure 7B).

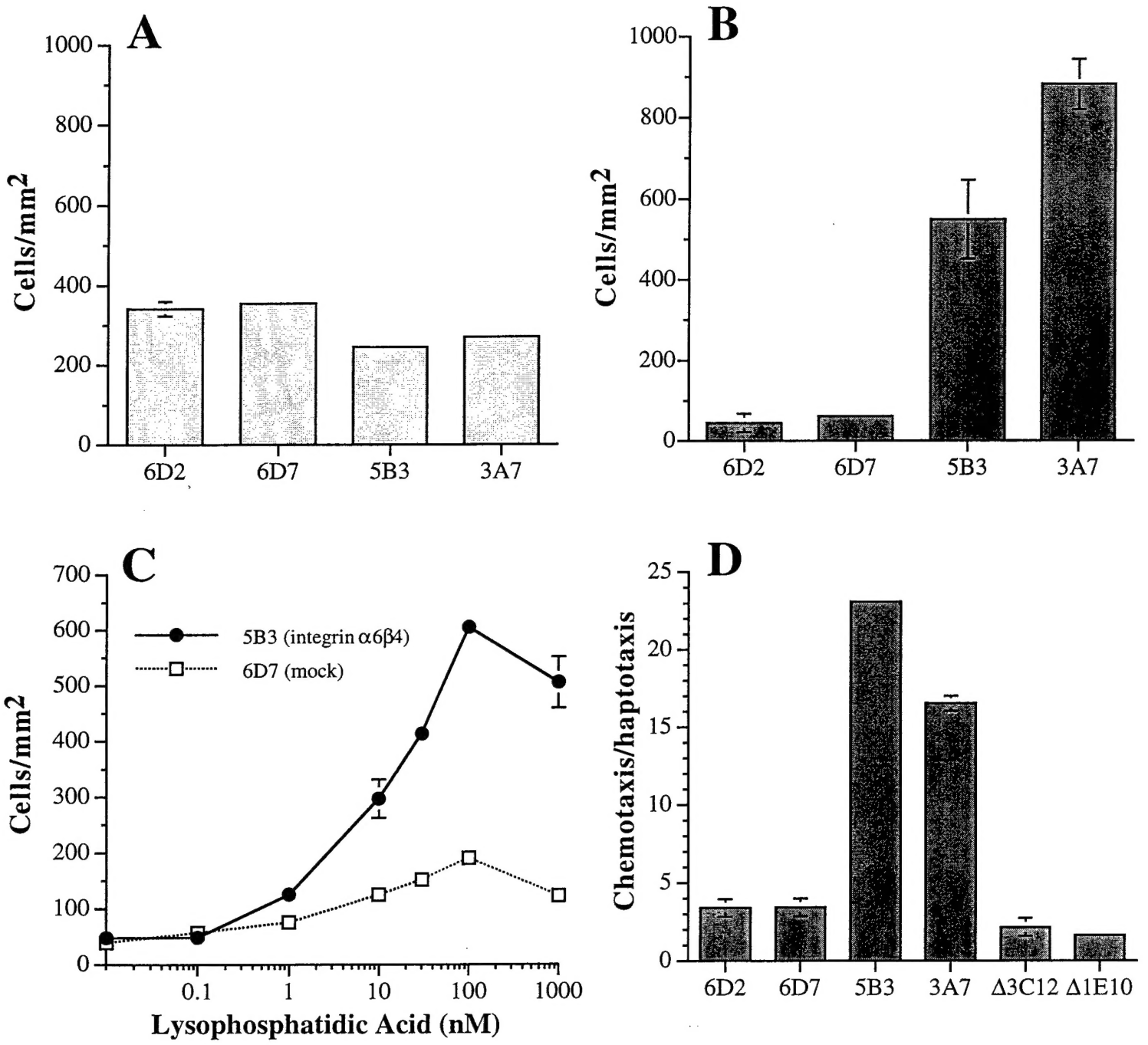
The necessity of cAMP-dependent PDE activity in the formation of lamellae was also assessed. IBMX had no effect on the morphology of the MDA/β4 transfectants in the absence of LPA (compare Figures 8A with 8B). However, IBMX-treated cells were unable to form the large, ruffling lamellae in response to LPA stimulation in comparison to untreated cells (compare Figures 8C with 8D). Quantitative analysis of these cell populations revealed that inhibition of PDE activity resulted in an approximate four-fold reduction in the lamellar area of LPA-stimulated MDA/β4 transfectants (Figure 8E).

Recently, we reported that α6β4 is necessary for the formation and stabilization of lamellae in clone A colon carcinoma cells plated on laminin-1 (31). If PDE activity is needed for lamellae formation as indicated by the above results, IBMX should inhibit the formation of lamellae in Clone A cells. To test this possibility, clone A cells were treated with IBMX or a solvent control and then plated onto laminin-1 for 45 min. The control cells formed large fan-shaped lamellae enriched in F-actin when plated on laminin-1 (Figure 9A,B) as we reported previously (31). In contrast, IBMX-treated cells formed small, immature lamellae with a marked reduction in F-actin content (Figure 9 C, D). Quantitative analysis of these images revealed that IBMX reduced the total lamellar area of clone A cells on laminin-1 by approximately 75% (629 ± 74 μm^2 for control vs. 164 ± 24μm^2 with IBMX). Interestingly, inhibition of PDE activity had no effect on the attachment or spreading of clone A cells on laminin-1 (Figure 9).

cAMP metabolism and PI3-K signaling are not directly linked in MDA-MB-435 cells: A possible relationship between cAMP metabolism and PI3-K signaling is of interest given our recent finding that α6β4 stimulates the preferential activation of phosphoinositide-3 OH kinase (PI3-K) and that this activity is required for invasion and the formation of lamellae. To determine if PI3-K activity is required for the cAMP-specific PDE activity we observed in the MDA/β4 transfectants, these cells were incubated in the presence of wortmannin, a specific inhibitor of PI3-K, prior to extraction and assay of PDE activity. As shown in Figure 10A, wortmannin had no effect on PDE activity in these cells and it did not inhibit the marked induction of PDE activity that we had observed in response to forskolin stimulation. The possibility also existed that cAMP influences the α6β4-mediated activation of PI3-K. To address this issue, we used the α6-specific mAb G0H3 to cluster α6 integrins on the MDA/β4 in the presence of the PDE inhibitor IBMX and forskolin. As shown in Figure 10B, mAb-mediated clustering of α6β4 in MDA/β4 transfectants activated PI3-K markedly compared to cells maintained in suspension, in agreement with our previous results (39). However, treatment of MDA/β4 transfectants with either IBMX or forskolin did not inhibit α6β4-mediated activation of PI3-K (Figure 10B). In fact, no inhibition of PI3-K was observed when both of these inhibitors were used in combination, a treatment that increases the $[cAMP]_i$ from 4 to 120 pmoles/10^6 cells (Figure 5).

Figure 1. Expression of the α6β4 integrin in MDA-MB-435 carcinoma cells stimulates chemotaxis but not haptotaxis. The migration of the MDA/β4 (5B3, 3A7) MDA/β4ΔCYT (Δ3C12, Δ1E10), and MDA/mock (6D2, 6D7) transfectants toward laminin (haptotaxis; A), 3T3 conditioned medium (chemotaxis; B) or LPA (chemotaxis; C, D) was assessed using a modified Boyden chamber. The lower wells of Transwell chambers were coated with either laminin (A) or conditioned media (B) or collagen I (C, D) and then $1x10^5$ (A, B) or $5x10^4$ (C, D) cells were placed in the upper chambers. After 4 hours at 37°C, cells that did not migrate were removed from the upper chamber with a cotton swab and cells on the opposite side of the membrane were fixed, stained, and quantified manually as described in the "Materials and Methods". (A) Haptotaxis toward laminin (B) Chemotaxis toward NIH 3T3 conditioned medium; (C) Dose response of MDA-MB-435 subclones 5B3 (β4 transfected; filled circles) and 6D7 (mock transfected; open squares) chemotaxis toward LPA. (D) Chemotaxis toward 100nM LPA where data are reported as fold increases over migration on collagen in the absence of LPA. Data are shown as mean ± standard deviation from triplicate determinations of a representative experiment.

Figure 1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6D7
5B3
3A7
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Cells/mm2
700
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500
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300
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100
0
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6D7 (mock)
0.1
1
10
100
1000
Lysophosphatidic Acid (nM)
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Chemotaxis/haptotaxis
25
20
15
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0
6D2
6D7
5B3
3A7
Δ3C12
Δ1E10

Figure 2. Inhibition of α6β4-stimulated migration by integrin-specific antibodies. MDA/β4 (5B3; A, B, gray bars) or mock transfectants (6D7; B, C; stippled bars) were incubated with the indicated function blocking mAbs for 30 min. prior to addition to a chemotaxis assay using 100nM LPA on collagen I (A) or a haptotaxis assay on laminin coated wells (B) as described in Figure 1. Non-specific mouse IgG was used as a negative control. Data are shown as the percent negative control ± standard deviation from triplicate determinations.

Figure 2

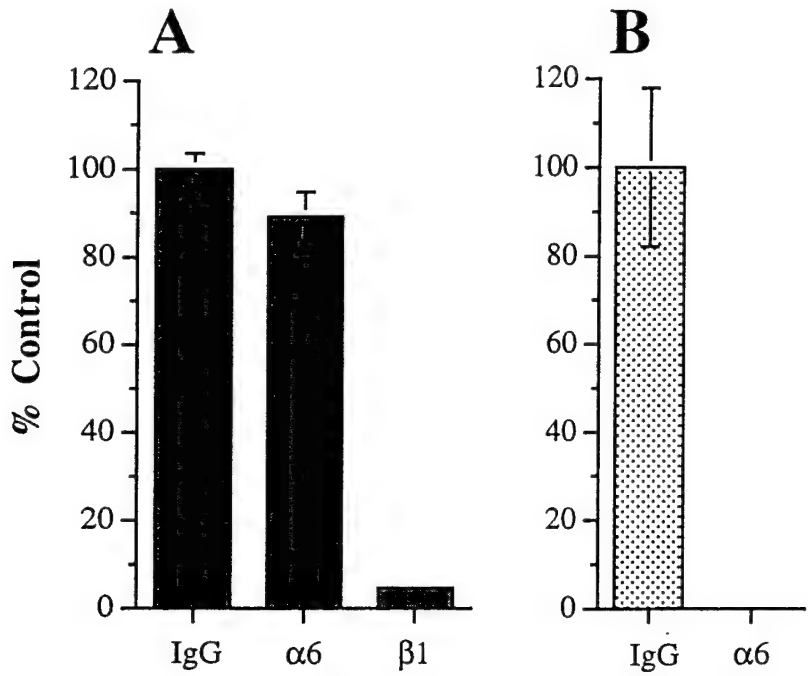
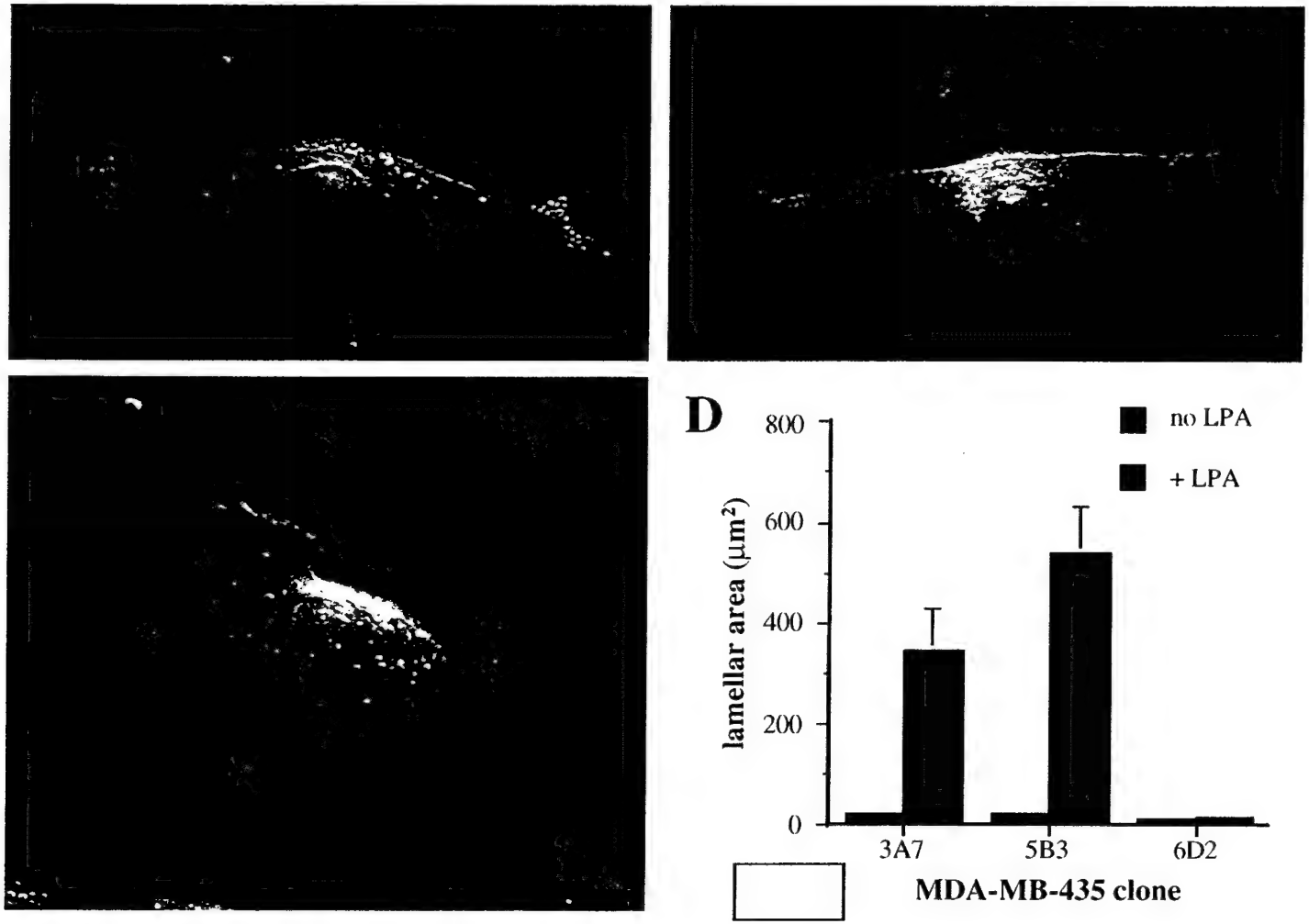


Figure 3. The α6β4 integrin is required for the LPA-dependent formation of lamellae in MDA-MB-435 cells. MDA/β4 (A, B) and mock transfectants (C) were plated onto coverslips that had been coated with 20μg/ml collagen I. Cells were allowed to adhere for 2hrs at 37°C and then treated with LPA for 5 min. (B, C) or left untreated (A). The cells were visualized using Nomarski DIC optics. Bar in A represents 10μm. In B, note the large lamellae that are formed in response to LPA stimulation of the MDA/β4 transfectants. (D) The effect of LPA on lamellar area was quantified using IPLab Spectrum imaging software. Data are shown as mean ± standard error in which $n > 20$.

Figure 3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400
200
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lamellar area (μm²)
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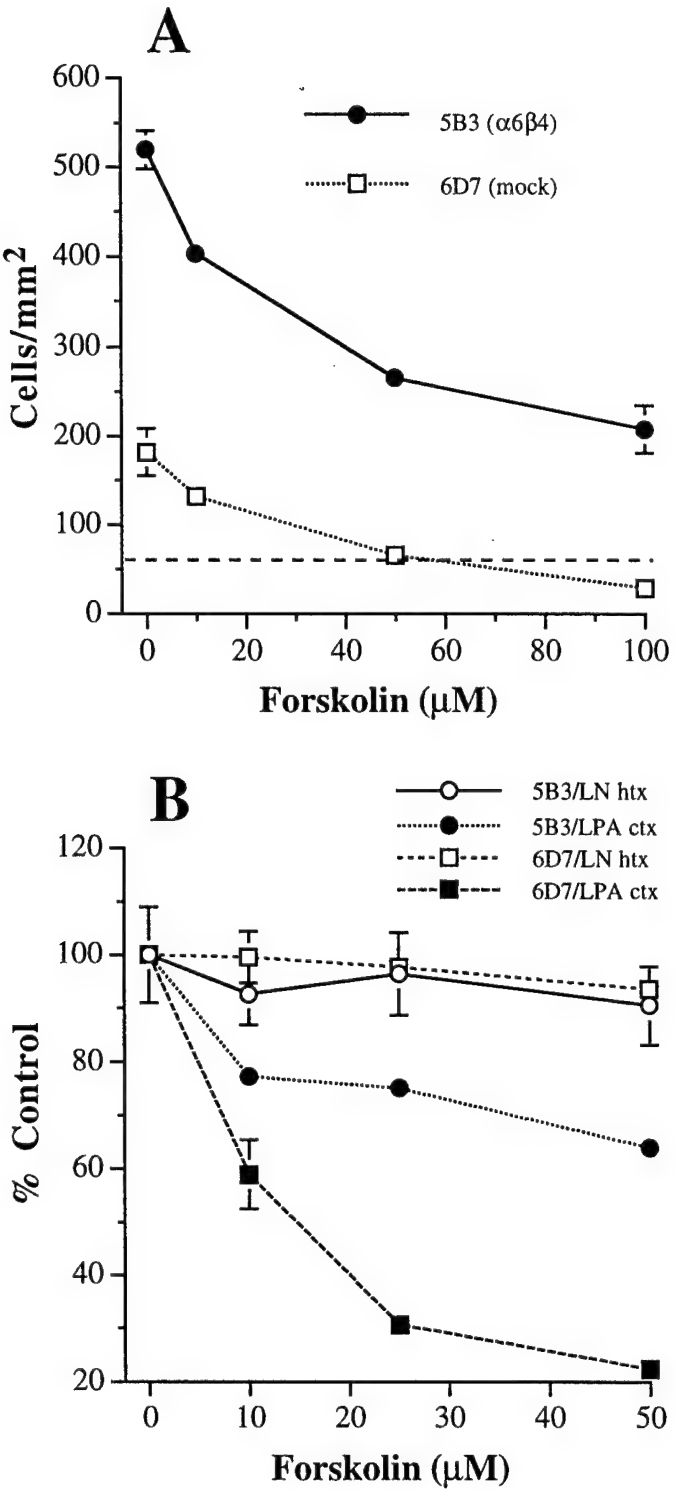
+ LPA
3A7
5B3
6D2
MDA-MB-435 clone

Figure 4. Forskolin stimulation of adenyl cyclase inhibits LPA mediated chemotaxis differentially in the MDA/β4 and mock transfectants. (A) MDA/β4 transfectants (5B3; solid circles) or mock transfectants (6D7; open squares) were treated with the indicated concentration of forskolin for 30 min. prior to addition to the upper wells of the Transwell chambers. Cells were assayed for LPA-mediated chemotaxis on collagen as described in Figure 1. The dashed line depicts the basal level of migration of both subclones in the absence of LPA. (B) In a separate experiment, the same cells were treated with forskolin for 30 min. prior to assaying for LPA chemotaxis (filled symbols) or laminin haptotaxis (open symbols). Data are reported as the percent migration of cells not treated with forskolin ± standard deviation of triplicate determinations. Representative experiment is shown.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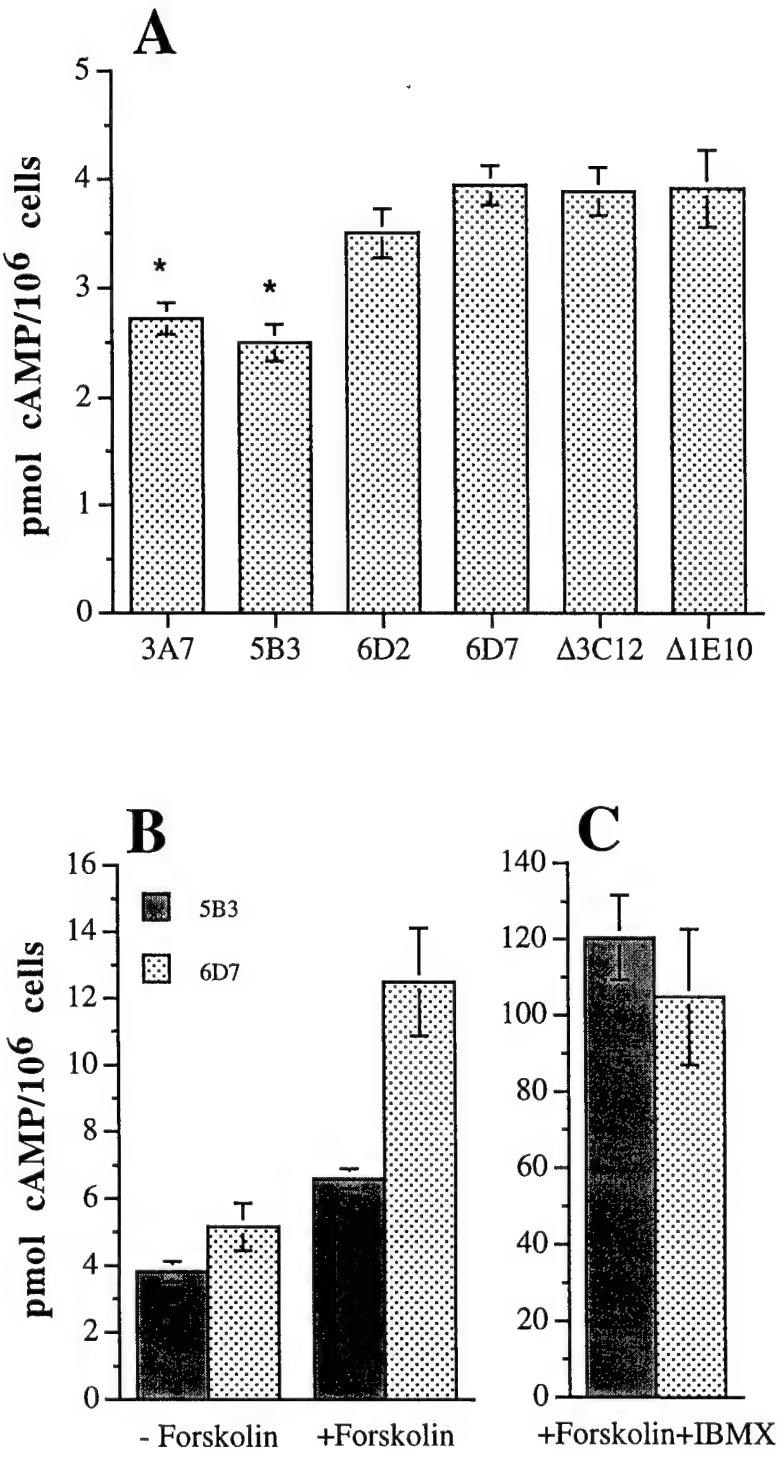
% Control
5B3/LN htx
5B3/LPA ctx
6D7/LN htx
6D7/LPA ctx
0
10
20
30
40
50
Forskolin (μM)

Figure 5. Intracellular cAMP content of the MDA-MB-435 transfectants. MDA/β4 (3A7, 5B3), MDA/β4-ΔCYT mutant (Δ3C12, Δ1E10) and MDA/mock transfectants (6D2, 6D7) were plated in DMEM plus 10% FCS. After 18 hrs., cells were harvested and cAMP content assessed using a cAMP EIA protocol as described in the "Materials and Methods." Data represent the mean of 10 sample determinations ± standard error. The difference in the cAMP levels between the MDA/β4 and the mock transfectants is significant ($p < 0.001$; denoted by asterisk), but the difference between the mock and the β4-ΔCYT transfectants is not ($p=0.2$). (B, C) Differential effects of forskolin stimulation on the $[cAMP]_i$ in the MDA/β4 and mock transfectants. The $[cAMP]_i$ was assayed in the 5B3 (solid bars) and 6D7 (stippled bars) clones plated on collagen type I and treated for 15 min. with 50μM forskolin (B) or with forskolin in conjunction with 1mM IBMX (C) as noted. Note that the MDA/β4 transfectants (5B3) are more resistant to a forskolin stimulated increase in cAMP than the mock transfectants (6D7). The inhibition of PDE activity with IBMX shown in C reveals that α6β4 expression results in an increase in phosphodiesterase activity and not a decrease in cAMP synthesis. Data from a representative experiment are shown as mean ± standard error.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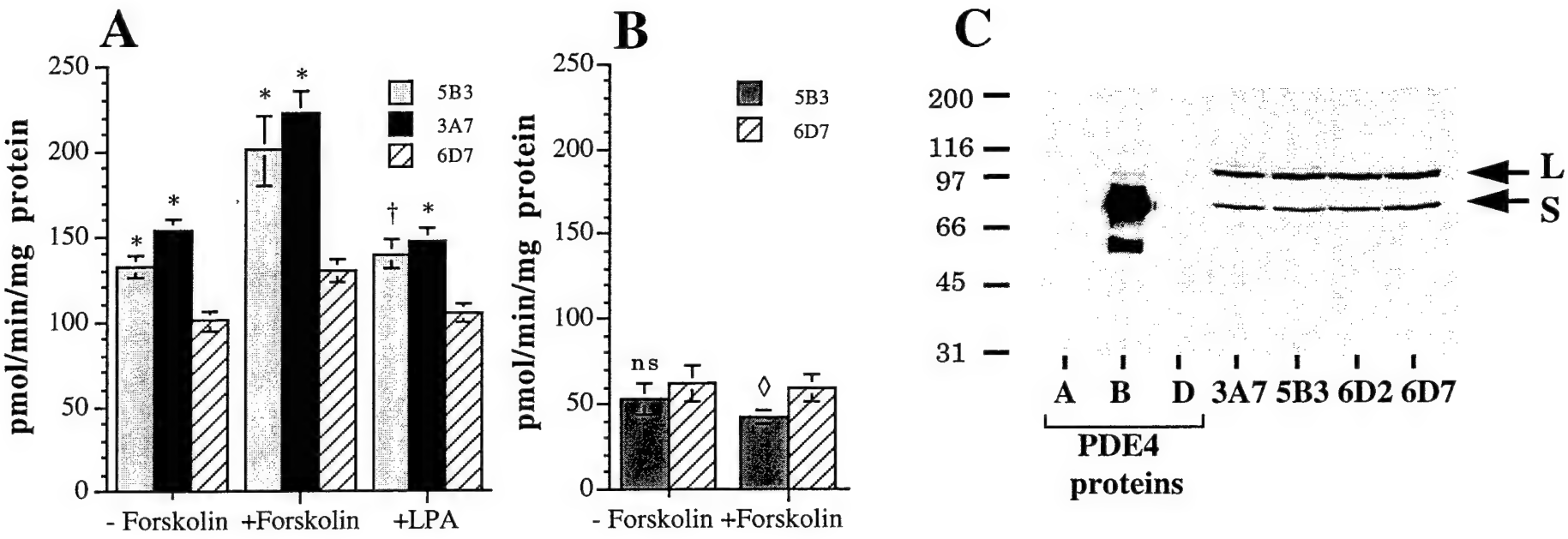
0
+Forskolin+IBMX

Figure 6. Assay of cAMP specific PDE activity of MDA-MB-435 transfectants. (A) MDA/β4 (3A7, 5B3) or mock transfectants (6D7) plated on collagen I were treated with 50 μM forskolin or 100nM LPA as noted. Cells were harvested and the cytosolic fraction was assayed for PDE activity as described in the "Materials and Methods." (*) p-value < 0.002; (†) p-value < 0.01 (B) Extracts from cells treated as in A were incubated with 100μM rolipram prior to assaying for PDE to determine how much of the activity in (A) constitutes cAMP-specific PDE (PDE type 4). Data represent mean ± standard error of 4 separate determinations (A, B). (ns) is not significant; (◊) p-value = 0.02 (C) Relative expression of PDE4B in the MDA-MB-435 transfectants. Extracts (40μg protein) obtained from the MDA/β4 (3A7, 5B3) and mock (6D2, 6D7) transfectants were resolved by SDS-PAGE and immunoblotted with a PDE4B specific Ab. Arrows in the right margin denote the long and short forms of PDE4B.

Figure 6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pmol/min/mg protein
250
200
150
100
50
0
5B3
3A7
6D7
- Forskolin
+Forskolin
+LPA
B
pmol/min/mg protein
ns
- Forskolin
+Forskolin
C
200
116
97
66
45
31
L
S
A
B
D
3A7
5B3
6D2
6D7
PDE4
proteins

Figure 7. A cAMP specific-PDE activity is required for the chemotactic migration and invasion of MDA-MB-435 cells. The MDA/β4 (5B3; squares) or mock transfectants (6D7; circles) were treated with the indicated concentration of IBMX (A) or 1mM IBMX (B) for 30 min. prior to their use in either an LPA chemotaxis assay (A) or a Matrigel chemoinvasion assay (B). Data represent mean ± standard deviation of triplicate determinations.

Figure 7

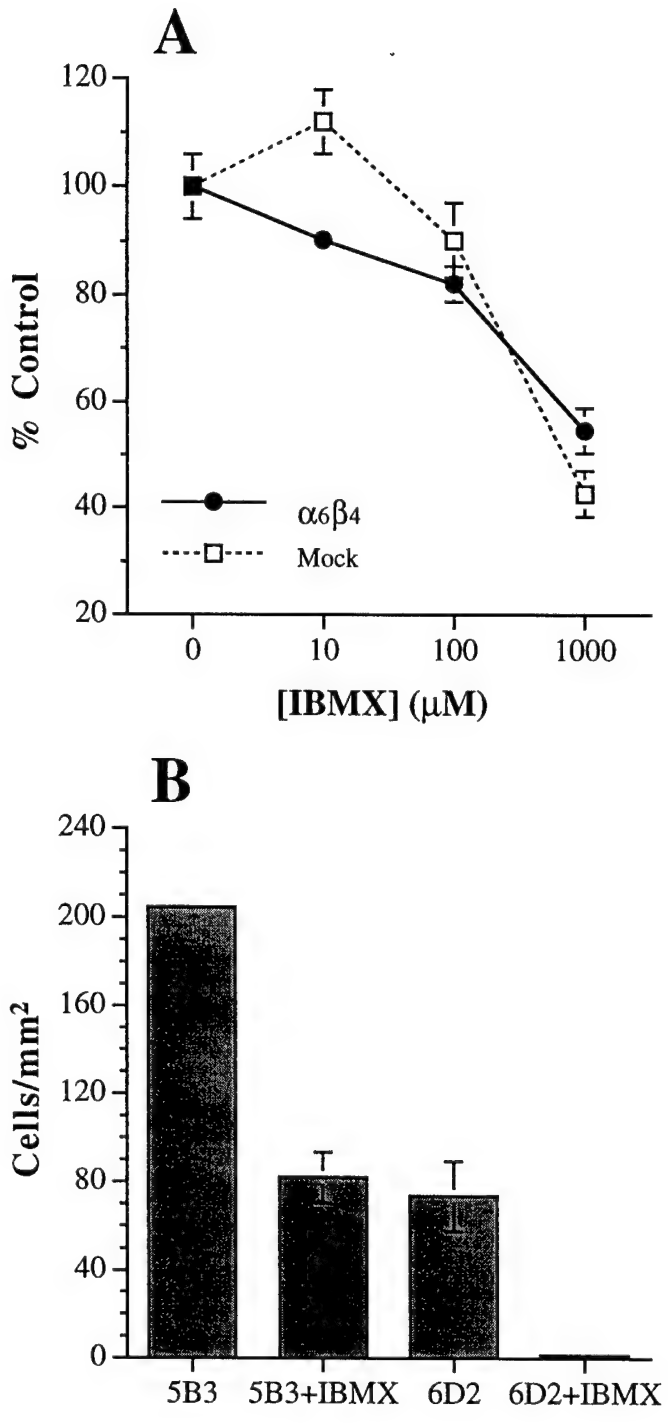
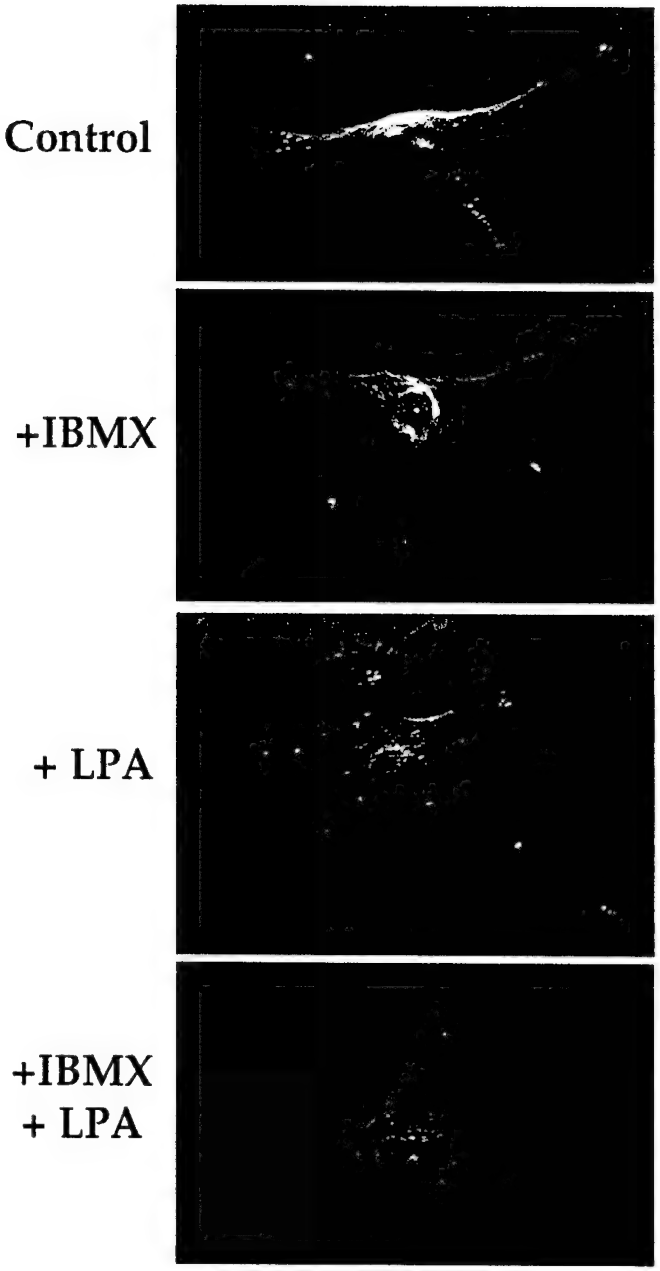

A
120
100
80
60
40
20
% Control
α6β4
Mock
0
10
100
1000
[IBMX] (μM)
B
240
200
160
120
80
40
0
Cells/mm²
5B3
5B3+IBMX
6D2
6D2+IBMX

Figure 8. cAMP specific-PDE activity is required for LPA-dependent formation of lamellae in MDA/β4 transfectants. The MDA/β4 transfectants (5B3) were plated on collagen coated coverslips. After 2 hours, the cells were either left untreated (A, C) or treated with 1mM IBMX (B, D) for 30 min. Subsequently, the cells were either left untreated (A, B) or treated with 100nM LPA for 5 min. (C, D). The cells were then fixed and visualized using Nomarski DIC optics. (E) The effect of LPA and IBMX on lamellar area was quantified using IPLab Spectrum imaging software. Bars represent standard error in which $n > 20$. Of note, IBMX inhibited the LPA-dependent formation of lamellae by 70%.

Figure 8


Control
+IBMX
+ LPA
+IBMX
+ LPA

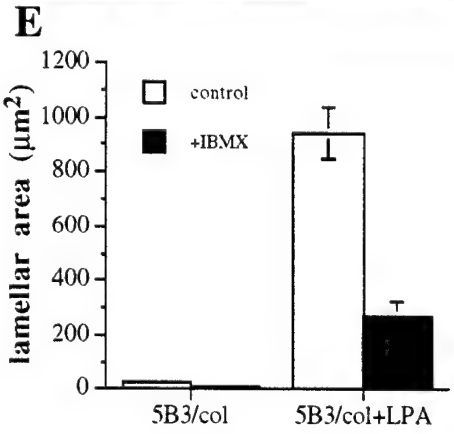

E
1200
1000
800
600
400
200
0
lamellar area (μm²)
control
+IBMX
5B3/col
5B3/col+LPA

Figure 9. Lamellae formation in Clone A colon carcinoma cells requires PDE activity. Clone A colon carcinoma cells were either treated with solvent alone (A, B) or 1mM IBMX in solvent (C, D) and then plated on laminin-1 coated coverslips. After 45 min., the cells were fixed and stained for F-actin using TRITC-phalloidin. Panels A and C, phase contrast images; panels B and D, fluorescence images. Bar in A represents 10μm.

Figure 9

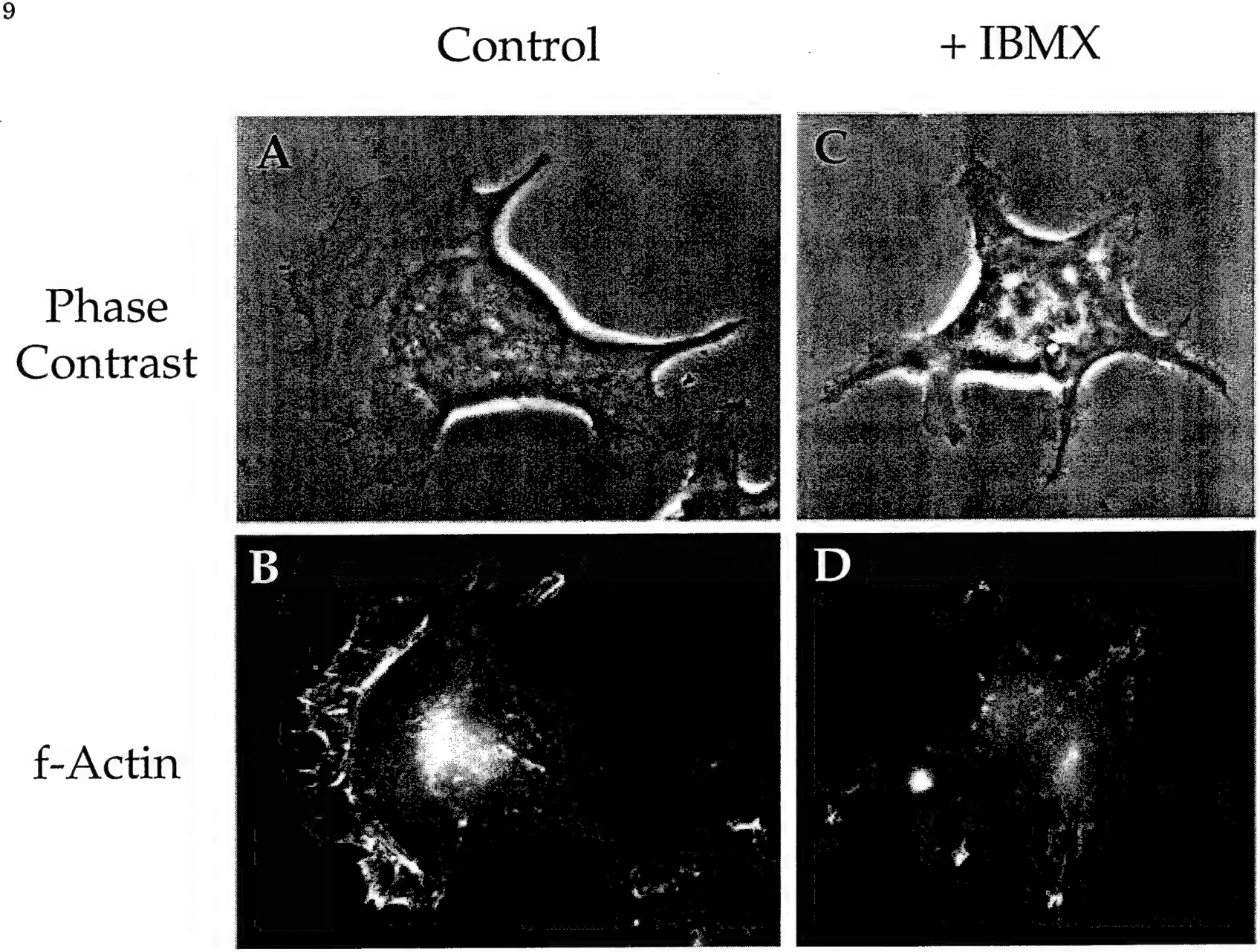
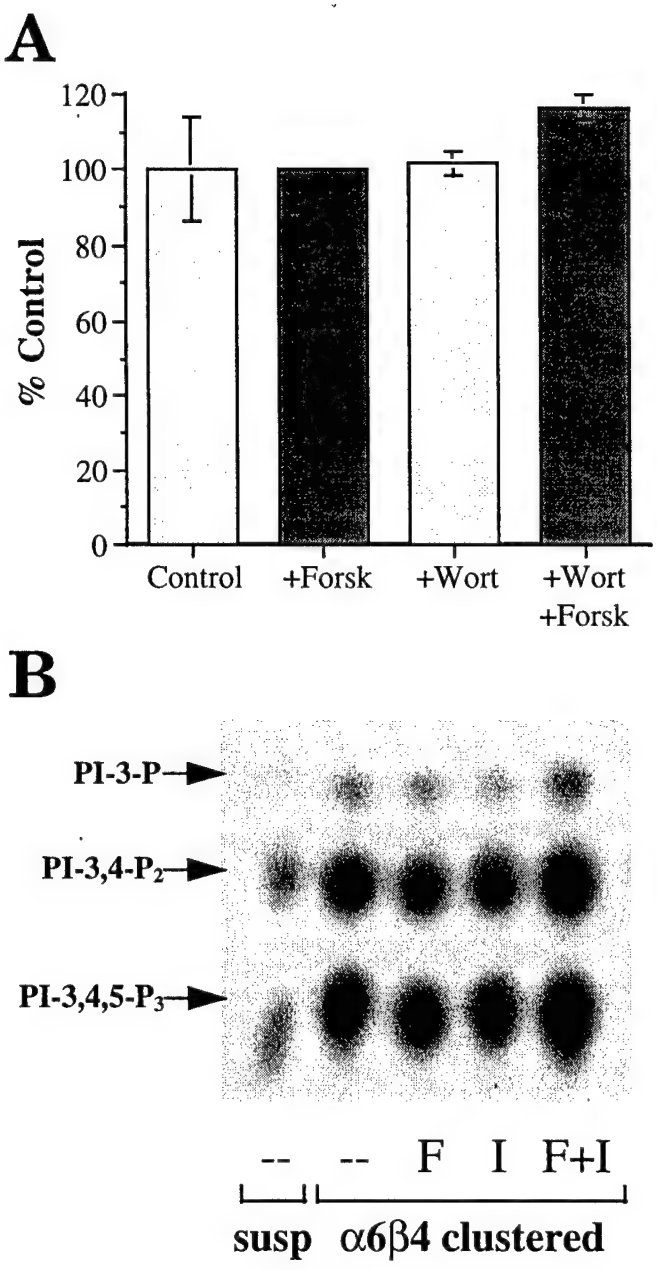


Figure 10. A) Evaluation of PI3-K involvement in PDE activity. The MDA/β4 transfectants were incubated for 30 minutes in the presence of either forskolin or wortmannin, or both in combination, prior to assay of PDE activity as described in Materials and Methods. B) Evaluation of the cAMP regulation of PI3-K activity. The MDA/β4 and mock transfectants were incubated in suspension with either forskolin or IBMX or both for 10 min. Subsequently, these cells were either maintained in suspension or incubated with a β4 integrin-specific antibody and allowed to adhere to anti-mouse IgG coated plates or laminin-1 coated plates for 30 min. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with the anti-phosphotyrosine mAb 4G10 and Protein A sepharose for 3hrs. After washing, the beads were resuspended in kinase buffer and incubated for 10 min. at room temperature. The phosphorylated lipids were resolved by thin layer chromatography. The D3-phosphoinositides are indicated by arrows.

Figure 10


A
120
100
80
60
40
20
0
% Control
Control
+Forsk
+Wort
+Wort
+Forsk
B
PI-3-P
PI-3,4-P2
PI-3,4,5-P3
--
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F
I
F+I
susp
α6β4 clustered

7) CONCLUSIONS: Recently, we established that the α6β4 integrin promotes carcinoma invasion (3, 31, 39). During Year 2 of this grant, we extended this observation by demonstrating that a major function of α6β4 is to stimulate the chemotactic migration of breast carcinoma cells, a function that is essential for invasion. This function is consistent with our previous finding that α6β4 is involved in the formation and stabilization of lamellae and filopodia (31). Importantly, the data presented here also provide evidence that α6β4 stimulates chemotaxis and lamellae formation by regulating the $[cAMP]_i$ by a mechanism that involves activation of a rolipram-sensitive, cAMP-specific PDE. Our finding that elevated $[cAMP]_i$ inhibits the formation of lamellae, chemotactic migration and invasion is in agreement with recent studies indicating that cAMP can function to inhibit or 'gate' specific signaling pathways (14, 16, 22). Furthermore, we show that the cAMP-mediated gate does not influence haptotaxis thus providing additional evidence that the signaling events that governing chemotaxis and haptotaxis differ (1, 19). Collectively, our results strengthen the hypothesis that α6β4 promotes carcinoma invasion through its ability to regulate signaling pathways required for migration. They also indicate that cAMP metabolism is likely to be an important factor in the regulation of carcinoma invasion and progression.

Although integrins can regulate a number of signaling pathways (4), their ability to influence cAMP metabolism has not been studied extensively. An earlier study, however, did provide evidence that the simultaneous engagement of β2 integrins and tumor necrosis factor (TNF) receptors decreases the $[cAMP]_i$ in neutrophils (29). Interestingly, the reduction in $[cAMP]_i$ observed in response to β2 integrin and TNF receptor engagement in neutrophils is similar to the level of $[cAMP]_i$ suppression that we observed in response to α6β4 expression (approximately 30%). This level of suppression of the total $[cAMP]_i$ is quite impressive given that localized gradients of $[cAMP]_i$ are probably required to facilitate chemotactic migration, as well as for other cell functions that are gated by cAMP. For example, localized gradients of cAMP have been implicated in regulating the direction of growth cone turning (40). It will be informative to monitor localized changes in the $[cAMP]_i$ at the leading edges of migrating cells as a function of time and to assess the influence of α6β4 on such concentration gradients.

A novel aspect of our study is the finding that integrins, and α6β4 in particular, can regulate the activity of a rolipram-sensitive, cAMP-specific PDE. This family of PDEs is defined as Type IV PDE (PDE 4) and consists of a number of structural variants (6). Because all of these variants hydrolyze cAMP with a K_m comparable to the $[cAMP]_i$, it is thought that tissue-specific expression and the state of activity of these variants are the major determinants of their responsiveness to extracellular stimuli (6). Indeed, the major focus of work in this area has been hormonal regulation of PDE activity. Regulation of PDE activity can occur rapidly in response to hormone stimulation through a mechanism that involves PKA-dependent phosphorylation of the enzyme (35, 36). In addition, long-term, hormonal stimulation can actually increase *de novo* synthesis of the cAMP-specific PDEs (6, 42). The data we obtained suggest that expression of α6β4 does not increase the expression of PDE4B, the predominant PDE variant expressed by MDA-MB-435 cells. For this reason, regulation of PDE4 activity by α6β4 expression may occur through a mechanism that involves PDE phosphorylation. Another possibility that has been proposed recently is that the sub-cellular localization of the cAMP-PDEs influences their function and activation (17). The possibility that α6β4 increases the association of PDE4 with either the plasma membrane or cytoskeleton is certainly attractive and could account, at least in part, for its ability to influence cAMP metabolism. Interestingly, LPA stimulation by itself had no effect on either PDE activity or the $[cAMP]_i$ in MDA-MB-435 cells. This observation reinforces our hypothesis that a major function of α6β4 is to release cAMP gating of LPA-stimulated chemotaxis.

In previous studies, we established that an important function of α6β4 in invasive carcinoma cells is its ability to stimulate the formation of lamellae (31). This function of α6β4 is highlighted by the observation in the present study that LPA was able to induce significant lamellae formation only in MDA-MB-435 cells that expressed α6β4 (Figure 3). Importantly, our finding that cAMP-PDE activity is necessary for lamellae formation promoted by α6β4expression implies that a localized suppression of the $[cAMP]_i$ plays an important role in controlling the signaling and cytoskeletal events that are required for lamellae formation. This hypothesis agrees with studies

that have shown an inhibitory effect of cAMP on the organization of the actin cytoskeleton (11, 13, 20, 21). Moreover, the formation of lamellae is a dynamic process that is linked to the mechanism of cell migration. Therefore, it is likely that temporal fluxes in the $[cAMP]_i$ regulated by α6β4 contribute to the chemotactic migration of carcinoma cells.

Of particular relevance to our work, Butcher and colleagues reported that cAMP is a negative regulator of leukocyte migration signaled through the classical chemoattractants (22). In this model, cAMP impedes or gates RhoA-mediated leukocyte integrin activation and adhesion. Our results support their conclusion that cAMP inhibits chemotactic migration. Importantly, we also provide evidence for an integrin-mediated mechanism of regulating the $[cAMP]_i$ to facilitate migration. Our findings support the work of the Butcher group because LPA is a potent activator of Rho (28) and, in fact, we have observed that the expression of a dominant-negative Rho can inhibit both LPA-stimulated chemotactic migration and invasion of MDA-MB-435 cells (O'Connor et al., preliminary observation). Thus, a possibility worth investigating is that LPA-mediated activation of Rho is gated by cAMP and that α6β4 releases cAMP gating by increasing cAMP-PDE activity and thereby enhances Rho activation. A likely target of Rho activation is the actin cytoskeleton (43), which is consistent with the reported effects of cAMP on the cytoskeleton (11, 13, 20, 21), as well as our demonstration that cAMP inhibits lamellae formation. Other integrins, especially the β1 integrins that mediate the adhesive interactions required for chemotactic migration, are another potential target of Rho (24, 33). It is worth noting in this context that expression of α6β4 has been shown to alter the function of collagen binding integrins in breast carcinoma cells (41).

The ability of α6β4to promote lamellae formation and carcinoma invasion is dependent upon its preferential activation of a PI3-K and Rac signaling pathway (39). Our current finding that the release of cAMP gating by α6β4 is also required for these events raised the issue of a possible link between cAMP and the PI3-K/Rac pathway. Such a link was suggested, for example, by the finding that the IL-2 dependent activation of PI3-K is inhibited by cAMP (27). In our experiments, however, pharamacological stimulation of cAMP levels had no effect on the ability of α6β4 to activate PI3-K even under conditions in which the $[cAMP]_i$ increased 30-fold over basal levels. Our data also indicate that PI3-K probably does not function upstream of cAMP-specific PDE because wortmannin did not inhibit the activity of this enzyme. We conclude from these findings that PI3-K and cAMP-specific phosphodiesterase function in tandem to promote lamellae formation and chemotactic migration but they are components of distinct signaling pathways.

An interesting finding in the present study is that the ability of α6β4 to stimulate chemotactic migration and suppress the $[cAMP]_i$ can be independent of the adhesive function of this integrin. Although the laminins are the only known matrix ligands for α6β4 (25), expression of this integrin also stimulated chemotactic migration on a collagen I matrix and this migration was not inhibited by an β4-function blocking mAb. The possibility that the effects of α6β4 expression on migration result from a decrease of α6β1 expression is discounted by the fact that expression of the α6β4 ΔCYT integrin had no effect on either chemotaxis, cAMP levels or PDE activity even though expression of this mutant eliminates α6β1 expression in these cells (37). The observation that the ability of α6β4 to promote chemotactic migration can be independent of its adhesive function is in agreement with several recent studies by our group and others that have revealed 'ligand-independent' functions for the α6 integrins in carcinoma cells (3, 5, 8). Insight into the possible mechanism of this phenomenon was provided by a recent study that demonstrated self-association of the β4 cytoplasmic domains, a process that could initiate intracellular signaling events independently of ligand binding (32). One important implication of these findings is that the ability of α6β4 to influence cAMP metabolism and stimulate the chemotactic migration of carcinoma cells need not be limited to sites of contact with laminin-containing matrices. This possibility is supported by the numerous studies that have implicated α6β4 as a major determinant of carcinoma invasion and progression (10, 34, 46).

In summary, we have demonstrated that the α6β4integrin can stimulate lamellae formation and chemotactic migration of invasive carcinoma cells by increasing the activity of a rolipram-

sensitive cAMP specific-PDE and lowering the $[cAMP]_i$. This cAMP specific-PDE functions in tandem with a PI3-K/Rac pathway, that is also regulated by $\alpha6\beta4$, and is required for carcinoma invasion and lamellae formation. The essence of our findings is that the $\alpha6\beta4$ integrin stimulates the chemotactic migration of carcinoma cells through its ability to influence key signaling events that underlie this critical component of carcinoma invasion.

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